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ORIGINAL ARTICLE

Characterization of the in vitro activity of AZD3409, a novel prenyl transferase inhibitor

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Abstract
Purpose AZD3409 is a novel DPTI that has potent activity against both FTase and GGTase-1. The in vitro inhibition profile of AZD3409 was characterized using three different cell lines: mouse embryogenic fibroblasts, transfected with H-Ras^{V12} (MEF), A549 cells (Ki4B-Ras mutation) and MCF-7 cells (no Ras mutation).
Methods Both cytotoxicity and levels of inhibition of farnesylation and geranylgeranylation were determined in different assays in relation to the concentration of AZD3409. Results were compared with those obtained with the first-generation FTase inhibitor lonafarnib or the GGTase-1 inhibitor GGTI-2147.
Results The mean IC_{50} for cytotoxicity of AZD3409 and lonafarnib was 510 and 15,200 nM in MEF cells, 10,600 and 2,740 nM in A549 cells and 6,170 and 9,490 nM in MCF7 cells, respectively. In these cells, the IC_{50} for FTase activity of AZD3409 ranged from 3.0 to 14.2 nM and of lonafarnib from 0.26 to 31.3 nM. The inhibiting activity of AZD3409 and lonafarnib on general protein farnesylation was comparable with the specific farnesylation levels of HDJ-2. In vitro geranylgeranylation of Rap1a could be inhibited by GGTI-2147 in all three cell lines, but only in MCF-7 cells by AZD3409. These results are in agreement with the IC_{50} values for GGTase-1 activity as the lowest IC_{50} for AZD3409 was found in the MCF-7 cell line.
Conclusions AZD3409 inhibits farnesylation to a higher extent than geranylgeranylation. Both inhibition of farnesylation and geranylgeranylation could not be correlated to the antiproliferative activity of the drug.

Keywords AZD3409 · Farnesyl transferase · Geranylgeranyl transferase · In vitro characterization

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Introduction

Ras proteins play an important role in cell growth processes. Mutation of the protein on position 12, 13 or 61 leads to impaired control resulting in aberrant cell growth. These mutations are found in roughly 30% of all human cancers [1]. Ras is activated through prenylation of the so-called C-terminal CAAX-box (C = cysteine, A = aliphatic amino acid, X = serine or methionine) [2]. Three isoforms of Ras exist, namely H-Ras, N-Ras, and K-Ras that is present in the splice variants Ki4A-Ras and Ki4B-Ras. H-Ras is exclusively farnesylated, while N- and K-Ras can both be farnesylated and geranylgeranylated [3]. Attempts to inhibit activation of mutated Ras as treatment option for cancer focused on the inhibition of farnesyl transferase (FTase) [4]. Many FTase inhibitors (FTIs) have been developed of

which at least six entered clinical trials [5, 6]. However, little activity has been observed in these trials, despite promising results in preclinical studies. A hypothesis is that Ki4B-Ras, the Ras protein that is found predominantly mutated in human cancer compared to the other isoforms, is alternatively geranylgeranylated when farnesylation is blocked [3]. Possibly, geranylgeranylated Ki4B-Ras can function in a similar manner as farnesylated Ki4B-Ras [7]. Therefore, inhibition of both farnesylation and geranylgeranylation may show superior antitumor activity above inhibition of farnesylation alone.

Lobell described the preclinical characterization of an inhibitor that blocks both FTase and geranylgeranyl transferase-1 (GGTase-1), a so-called dual prenyl transferase inhibitor (DPTI) [8]. Again, promising results were obtained when the drug was administered to human cancer cell lines. Ki4B-Ras prenylation was blocked, and significantly higher levels of apoptosis were obtained with a DPTI relative to an FTI alone. Administration of the DPTI in mice, however, was lethal. A structurally similar but less potent DPTI is L-778123. The IC_{50} of L-778123 for FTase and GGTase-1 activity is 2 and 98 nM, respectively [9]. This drug was well tolerated in dogs, and inhibition of both HDJ-2 farnesylation and Rap1a geranylgeranylation (although $\leq$5%) was shown in lysates of PBMCs [10]. However, unprenylated Ki4B-Ras could not be detected.

AZD3409 is a novel DPTI that also has good inhibition characteristics against prenyl transferases. It is administered as a "double" prodrug and is rapidly converted to a thiol-ester prodrug. This is referred to as AZD3409-ester. The AZD3409-ester can penetrate into the cell where a thiol-acid moiety is formed under the action of intra-cellular esterases. This is the active drug and referred to as AZD3409-acid. The IC_{50} of AZD3409-acid for FTase and GGTase-1 isolated enzymes is 1 and 30 nM, respectively, using Ki4B-Ras as a substrate (unpublished data). It has been shown that AZD3409 significantly inhibits the growth of H-Ras-transformed mouse 3T3 fibroblasts (Hras5; IC_{50} = 49 nM) and LoVo human colorectal cells with mutant Ki4B-Ras (IC_{50} = 390 nM) [11]. Additionally, inhibition of FTase activity and Ras prenylation in Hras5 cells was shown, with IC_{50}'s of 3 and 100 nM, respectively (unpublished data). In models of urothelial carcinoma, treatment with AZD3409 resulted in significant growth inhibition of 253 J-BV, RT-112, T24 and J82 urothelial cells ($IC_{50} \sim 5$ μM) [12, 13]. Also in breast and ovarian cancer cell lines, dose-dependent growth inhibition with IC_{50} values in the low micromolar range was observed after treatment with AZD3409 [14].

In this paper, we have extended the characterization of the in vitro activity of AZD3409 to inhibit farnesylation and geranylgeranylation using different assays in three different cell lines expressing mutated Ki4B-Ras, mutated H-Ras or wild-type Ras and compared these activities with those of the FTI lonafarnib and the GGTase-1 inhibitor GGTI-2147.

Materials and methods

Compounds

AZD3409 (AstraZeneca Pharmaceuticals, Alderley Park, Cheshire, United Kingdom) was dissolved in DMSO at a concentration of 100 mg/ml. A fresh stock solution was prepared once a month and stored at −20°C. Working solutions were prepared freshly before each experiment. Lonafarnib (Schering Plough Research Institute, Kenilworth, NJ, USA) and GGTI-2147 (Calbiochem, Merck, Darmstadt, Germany) were also dissolved in DMSO at a concentration of 100 mg/ml. When comparative experiments with AZD3409 and lonafarnib or GGTI-2147 were performed, equimolar drug concentrations were used.

Recombinant H-RasWt, H-RasCVLL and GGTase-1 originated from Invitrogen (Carlsbad, CA, USA). Recombinant FTase and mouse anti-HDJ-2 antibodies were obtained from Calbiochem. Tritium-labelled farnesyl-pyrophosphate ([^{3}H]-FPP) and tritium-labelled geranylgeranyl-pyrophosphate ([^{3}H]-GGPP) were purchased at American Radiolabeled Chemicals Inc. (St Louis, MO, USA). Goat anti-Rap1a and donkey anti-goat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA,USA). Goat anti-mouse originated from Dako Cytomation (Carpinteria, CA, USA).

All other reagents were of analytical grade and obtained from standard suppliers.

Cell lines

MCF-7 human breast cancer cells were cultured under standard conditions in RPMI 1640 medium supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin sulphate and 10% (v/v) foetal bovine serum. Immortalized mouse embryonic fibroblasts (MEF) stably transfected with H-Ras^{V12} were a kind gift of T. Brummelkamp (The Netherlands Cancer Institute). MEFs and the A549 human lung cancer cell line were cultured in DMEM supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin sulphate and 10% (v/v) foetal bovine serum.

Cytotoxicity studies

Cells were seeded in 96-well plates on day 1 and treated on day 2 with AZD3409 or lonafarnib at concentrations ranging from 1 nM to 100 μM. After a 3-day incubation (without replenishment of the drug), cells were washed and stained with sulforhodamine B [15]. The extinction

was measured at 540 nm and inversely correlated with the cytotoxicity. The data were fitted to a sigmoidal concentration–response curve with a variable slope using Graphpad Prism software (version 4.02 for Windows, GraphPad Software, San Diego California USA). The minimal and maximal survival fractions were set at 0 and 1, respectively. The steepness of the slope is described by the Hill coefficient (γ). The IC_{50} was calculated by the software.

Filter-binding assay

Cells were seeded in 14-cm petri dishes on day 1 and treated with AZD3409 or lonafarnib on day 3. After 24 h, cells were scraped and the pellets were stored at −70°C. For FTase activity measurements, the cell pellets were lysed with CHAPS-buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1% CHAPS and protease inhibitors) and the protein concentration was immediately assessed using a Bradford assay. A concentration of 50 µg of total protein was incubated with 4 µM H-RasWt and 0.5 µM [^{3}H]-FPP for 60 min at 37°C. The reaction was stopped by adding 10% HCl in ethanol. Proteins were precipitated with 100% ethanol, and the suspension was filtrated. The filters were incubated in Solvable^{TM} for 20 min at 60°C, Ultima Gold counting liquid was added, and the radioactivity was determined using liquid scintillation counting. The reaction was performed in quadruplicate. Blanks consisted of the same reaction mixture omitting the H-RasWt. For functional GGTase-1 studies with isolated recombinant GGTase-1 enzyme, the activity was determined by a similar method; H-RasWt, [^{3}H]-FPP and lysate were replaced by H-Ras-CVLL, [^{3}H]-GGPP and recombinant GGTase-1, respectively.

The data were fitted to a sigmoidal concentration–response curve with a variable slope using Graphpad Prism software. The minimal and maximal FTase activities were set at 0 and 100%, respectively.

Protein farnesylation analysis

Previously, we found that lysis of cells leads to activation of a protease that cleaves at the N-terminal site of farnesylated cysteines, thereby producing farnesyl-methylcysteine or FmC [16]. The concentration of this peptide might serve as a surrogate endpoint for the biological effect of FTIs. Cells were seeded in 14-cm petri dishes on day 1 and treated with AZD3409 or lonafarnib on day 2. After 3 days of incubation, cells were scraped and the pellets were stored at −70°C. The cell pellets were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl) and immediately analysed. Quantification of FmC was carried out with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [16]. The protein concentration was determined using a Bradford assay. The FmC concentration data were corrected for the total protein concentration and fitted to a sigmoidal dose–response curve with a variable slope using Graphpad Prism software. To assess IC_{50} values, the minimal and maximal FmC concentrations with regard to the FmC concentration in untreated cells were set at 0 and 100%, respectively.

HDJ-2 farnesylation assay

Cells were seeded in 14-cm petri dishes on day 1 and treated with AZD3409 or lonafarnib on day 2. After 3 days of incubation, cells were scraped and the pellets were stored at −70°C. The cell pellets were lysed with CHAPS-buffer, and the protein concentration was determined using a Bradford assay. A concentration of 10 µg of total protein was separated on a 10% acrylamide gel. Proteins were blotted onto a nitrocellulose membrane and incubated with mouse anti-HDJ-2 (1st antibody) and goat anti-mouse (2nd antibody). Bands were visualized with enhanced chemiluminescence. The intensity of the bands was evaluated by direct observation.

Rap1a geranylgeranylation assay

Cells were seeded in 8-cm petri dishes and treated with AZD3409 on day 2 or 3. After a 1-day incubation, cells were scraped and the pellets were stored at −70°C. The cell pellets were lysed with RIPA buffer (100 mM Tris–HCl, 2 mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholic acid, 0.5% (w/v) Nonidet P-40; pH 8.3 and protease inhibitors), and the protein content was determined using a Bradford assay. A concentration of 50 µg of total protein was separated on a 10% acrylamide gel. Proteins were blotted onto a nitrocellulose membrane and incubated with a goat anti-Rap1a antibody that specifically recognizes unprenylated Rap1a (1st antibody) and anti-goat-HRP (2nd antibody) [10]. Bands were visualized with enhanced chemiluminescence.

Results

Inhibition of tumour cell proliferation

The ability of AZD3409 and its metabolites to inhibit tumour cell growth was investigated using a mouse embryogenic fibroblast cell line stably transfected with the H-RasV12 oncogene (MEF). In addition, proliferation studies were performed in the human lung tumour cell line A549, which is known to express oncogenic Ki4B-Ras and in the human breast tumour cell line MCF-7, which

expresses wild-type Ras. After an exposure of 72 h to AZD3409, cell growth of all three cell lines was inhibited in a dose-dependent manner (Fig. 1). The IC_{50} values are tabulated in Table 1. AZD3409 was a more potent inhibitor of cell growth in MEF and MCF-7 cell lines. In MEF cells, AZD3409 was approximately 30 times more cytotoxic than lonafarnib, while in MCF-7 cells, a difference of a factor of 1.5 was found. A549 cells were more sensitive to lonafarnib. In these cells, the IC50 of lonafarnib was about 4 times lower than the IC50 of AZD3409.

Inhibition of FTase activity

Farnesylation was monitored by employing three different assays. First, we determined the enzyme activity in a filter-binding assay. The cells were treated with increasing concentrations of AZD3409 or lonafarnib. The obtained inhibition curves are shown in Fig. 2, and the IC_{50}'s are outlined in Table 1. AZD3409 was a more potent FTase inhibitor than lonafarnib in both MEF and A549 cells; however, the difference in IC_{50} was not significant.

To determine whether inhibition of FTase resulted in inhibition of protein farnesylation, we analysed the amount of FmC that was formed after lysis of treated cells. Proteins that are farnesylated contain a common CAAX sequence at their C-terminal site. After farnesylation of the protein, this sequence is proteolyzed and carboxymethylated at the cysteine [17]. Lysis of the cells results in the activation of a proteolytic enzyme that cleaves before the C-terminal cysteine resulting in the formation of FmC. We have developed a method to quantify the amount of FmC in cancer cells and peripheral blood mononuclear cells (described elsewhere [16]). The concentration of FmC decreased from 0.66 to 0.19 pmol/mg protein. It was assumed that the FmC concentration in the cell lysates could be used as a parameter for the level of total protein farnesylation in the cell cytosol. Figure 3 shows the FmC concentration (relative to the basal FmC level in untreated cells) versus the drug concentration. The exposure time was 72 h without replenishment of the drug. In all cell lines, inhibition of farnesylation could be quantified (Table 1). The IC_{50} in MEF cells was 5 times lower for lonafarnib than for AZD3409, but the difference was not significant. Also, in A549 cells, there was no significant difference in potency of the drugs. In MCF-7 cells, a decrease in the FmC concentration was not observed until concentrations above 100 nM AZD3409. On the contrary, lonafarnib induced 50% inhibition of farnesylation at low nanomolar concentrations.

Besides total farnesylation levels, we also determined the farnesylation level of a specific protein, which can be used as a biomarker. HDJ-2 (or MDJ-2 in mouse cells) is a chaperone protein that is exclusively farnesylated. The farnesylated form has a lower mobility than its unprenylated form when separated on an SDS–PAGE gel. In a comparison of potential biomarkers for FTI inhibition, HDJ-2 was shown to be a very suitable marker in cells [18]. We treated the three cell lines with a dilution series of 4800 nM AZD3409 or lonafarnib. Representative western blots of concentrations up to 1200 nM are shown in Fig. 4. The IC_{50} values for HDJ-2 farnesylation in A549 and MCF7 cells

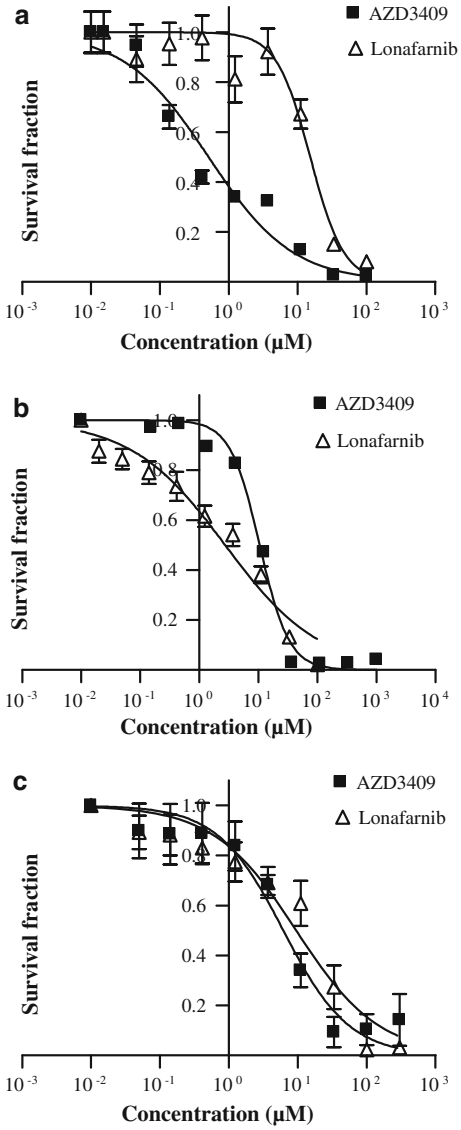

Fig. 1 Cell survival after treatment with either AZD3409 or lonafarnib at increasing concentrations (mean ± SEM). Data were fitted to a sigmoidal dose–response curve with a variable slope. The maximal fraction of surviving cells (F_{max}) was set at 1, the minimal fraction was set at 0 (F_{min}). The following equation was used to describe the data: $y = (F_{min} + (F_{max} - F_{min}))/(1 + 10\exp(\log IC_{50} - x)\gamma)$. y = cell survival relative to the basal level in untreated cells; x = logarithm of the drug concentration (nM). **a** MEF cells; AZD3409: $IC_{50} = 0.510$, $\gamma = -0.70$; lonafarnib: $IC_{50} = 15.2$, $\gamma = -1.8$. **b** A549 cells; AZD3409: $IC_{50} = 10.6$, $\gamma = -1.7$; lonafarnib: $IC_{50} = 2.74$, $\gamma = -0.54$. **c** MCF7 cells; AZD3409: $IC_{50} = 6.17$, $\gamma = -0.91$; lonafarnib: $IC_{50} = 9.49$, $\gamma = -0.72$

Table 1 IC_{50} values of AZD3409 and lonafarnib/GGTI-2147 (represented as mean and [95% confidence interval])

Parameter/inhibitor	IC_{50} (nM)		
	MEF	A549	MCF-7
Cytotoxicity (72-h exposure)			
AZD3409	510 [380–690]	10,600 [9,800–11,300]	6,170 [5,100–7,550]
Lonafarnib	15,200 [11,400–20,300]	2,740 [2,000–3,700]	9,490 [7,700–11,700]
FTase activity (24-h exposure)			
AZD3409	12.6 [3.3–48.3]	3.0 [0.8–11.1]	14.2 [6.1–32.8]
Lonafarnib	31.3 [9.0–109]	16.6 [1.7–163]	0.26 [0.074–0.91]
Protein farnesylation (24-h exposure)			
AZD3409	569 [190–1,700]	350 [114–1,080]	12,900 [587–2.8 × 10^6]
Lonafarnib	99 [27–359]	524 [270–1,020]	13.5 [3.90–46.8]
HDJ-2 farnesylation (72-h exposure)			
AZD3409	1,200	150	5,000
Lonafarnib	300	300	19
GGTase activity (24-h exposure)			
AZD3409	8,200 [4,471–15,039]	6,433 [3,333–12,418]	91.9 [5.8–1,470]
GGTI-2147	212 [21.2–2,117]	15,517 [1.3–1.9 × 10^8]	63.2 [0.7–5,601]

(Table 1) are in agreement with the FTase data. AZD3409 appeared to be a slightly more potent inhibitor of HDJ-2 farnesylation than lonafarnib in A549 cells. However, in the MCF7 cell line, HDJ-2 could not be inhibited by AZD3409 up to micromolar concentrations. Based on additional experiments with higher concentrations AZD3409 m, the IC_{50} is estimated at 5 μM. In MEF cells, we found that lonafarnib is a fourfold more potent inhibitor of farnesylation of MDJ-2 than AZD3409.

Inhibition of GGTase-1 activity

AZD3409 is also a moderately potent inhibitor of GGTase-1. It is hypothesized that this feature will increase the antitumour activity of the drug, since it may also inhibit isoprenylation of Ki4B-Ras, the Ras protein that is found predominantly mutated in human cancer. To determine the capability of AZD3409 to inhibit GGTase-1, we developed two assays: a filter-binding assay to determine GGTase-1 activity and a western blot for the Rap1a protein. Rap1a is a small GTP-binding protein that has a function in intracellular signalling and cytoskeletal control and that is exclusively geranylgeranylated. As a positive control, we used GGTI-2147, a GGTase-1 inhibitor. The obtained GGTase-1 inhibition curves are given in Fig. 5, and the IC_{50}'s are outlined in Table 1. Geranylgeranylation in the MCF-7 cell line was most sensitive to AZD3409 treatment. GGTI-2147 was a more potent GGTase-1 inhibitor than AZD3409 in all three cell lines; however, the difference in IC_{50} was not significant due to the high inter-experiment variability.

Figure 6 shows a western blot of the three cell lines, which were treated with AZD3409, GGTI-2147 and lonafarnib at micromolar concentrations. The anti-Rap1a antibody that was applied only recognizes the unprenylated form of Rap1a [10]. We investigated an exposure time of 24 h. After incubation with AZD3409, unprenylated Rap1a could only be detected in the MCF-7 cell line, while GGTI-2147 inhibited geranylgeranylation in all three cell lines. As expected, lonafarnib did not inhibit Rap1a geranylgeranylation.

Discussion and conclusion

We characterized the in vitro cytotoxicity and inhibition of farnesylation and geranylgeranylation activity of AZD3409 in three different cell lines: mouse embryogenic fibroblasts, transfected with human H-Ras^{V12} (MEF), A549 human lung cancer cells (K-Ras mutation) and MCF-7 human breast cancer cells (no Ras mutation) and compared the results with those obtained with the FTI lonafarnib and the GGTase-1 inhibitor GGTI-2147.

The cell survival experiments showed that AZD3409 is a more potent inhibitor of cell growth than lonafarnib in two of the three cell lines. Surprisingly, all IC_{50} values were in the micromolar range, while the intrinsic activities against FTase and GGTase-1 are in the low nanomolar range. Ras proteins have a half life of approximately 1 day, which means that after 3 days still 13% of farnesylated Ras will be present to drive cell growth [19]. Longer incubation studies may therefore give a better estimation of the toxicity. Furthermore, we observed that the MEF cells were only moderately sensitive to lonafarnib treatment. This is in accordance with the results of Ashar and co-workers. They

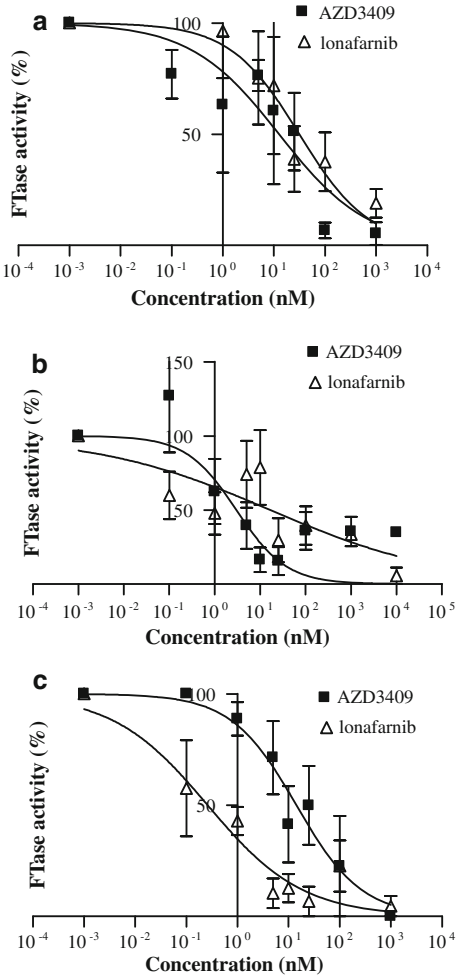

Fig. 2 FTase activity in cells after treatment with either AZD3409 or lonafarnib at increasing concentrations (mean ± SEM). Data were fitted to a sigmoidal dose–response curve with a variable slope. The maximal effect (E_{max}) was set at 100%, the minimal effect was set at 0% (E_{min}). The following equation was used to describe the data: $y = (E_{min} + (E_{max} - E_{min}))/(1 + 10\exp(\log IC_{50} - x)\gamma)$. y = FTase activity relative to the basal level in untreated cells; x = logarithm of the drug concentration (nM). **a** MEF cells; AZD3409: $IC_{50} = 12.3$, $\gamma = -0.50$; lonafarnib: $IC_{50} = 31.3$, $\gamma = -0.64$. **b** A549 cells; AZD3409: $IC_{50} = 3.0$, $\gamma = -0.79$; lonafarnib: $IC_{50} = 16.6$, $\gamma = -0.23$. **c** MCF7 cells; AZD3409: $IC_{50} = 14.2$, $\gamma = -0.68$; lonafarnib: $IC_{50} = 0.26$, $\gamma = -0.47$

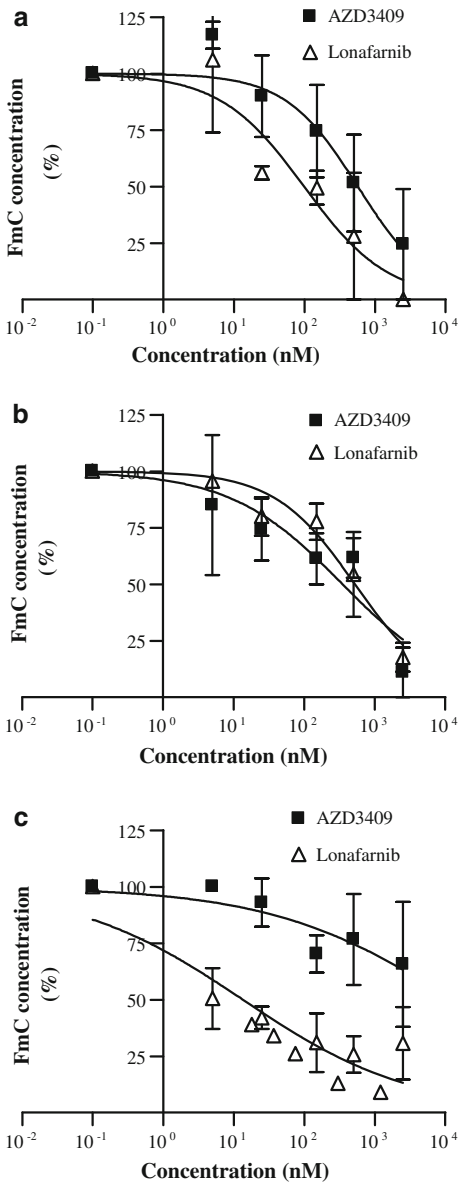

Fig. 3 Relative FmC concentration in cell lysates after treatment with either AZD3409 or lonafarnib at increasing concentrations (mean ± SEM). The concentration in untreated cells was set at 100%. Data were fitted to a sigmoidal dose–response curve with a variable slope. The maximal effect (E_{max}) was set at 100%, the minimal effect was set at 0% (E_{min}). The following equation was used to describe the data: $y = (E_{min} + (E_{max} - E_{min}))/(1 + 10\exp(\log IC_{50} - x)\gamma)$. y = FmC concentration relative to the basal level in untreated cells; x = logarithm of the drug concentration (nM). **a** MEF cells; AZD3409: $IC_{50} = 569$, $\gamma = -0.85$; lonafarnib: $IC_{50} = 99$, $\gamma = -0.73$. **b** A549 cells; AZD3409: $IC_{50} = 350$, $\gamma = -0.55$; lonafarnib: $IC_{50} = 524$, $\gamma = -0.77$. **c** MCF7 cells; AZD3409: $IC_{50} = 12900$, $\gamma = -0.33$; lonafarnib: $IC_{50} = 13.5$, $\gamma = -0.36$

found that cell lines that contain wild-type p53 are more sensitive to lonafarnib treatment than cells that express mutated p53 [20]. The MEF cells in our experiments are p53 negative, while MCF7 and A549 cells both harbour the wild-type p53 gene. This correlation between p53 status and cytotoxicity could not be obtained for AZD3409. Probably, the mechanism of action of AZD3409 is not mediated by p53.

Additionally, we did not find a correlation between mutation status and sensitivity to AZD3409. This is in accordance with previous reports with FTIs other than AZD3409, which demonstrated that the sensitivity of a cell line to an FTI does not depend on the presence of a mutated *Ras* gene [21–23]. Possibly, mutation of Ras plays only an important role in the initiation of tumour development. Later genetic events might adopt tumour growth control

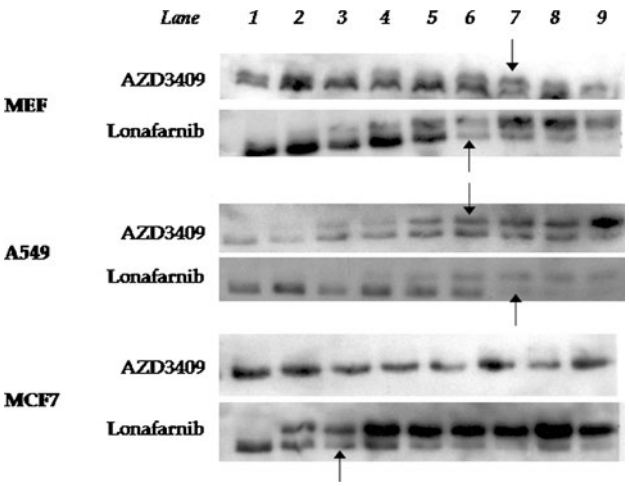

Fig. 4 Representative western blots of HDJ-2. Appearance of two equally intense bands at 44 kDa was determined as the concentration at which 50% of the farnesylation of HDJ-2 was inhibited. The drug concentrations were as follows: *Lane 1* mock-treated; *Lane 2* 9 nM; *Lane 3* 19 nM; *Lane 4* 38 nM; *Lane 5* 75 nM; *Lane 6* 150 nM; *Lane 7* 300 nM; *Lane 8* 600 nM; *Lane 9* 1,200 nM. The *arrows* indicate the IC_{50} that was determined by direct observation of the respective blots. Experiments were performed in triplicate

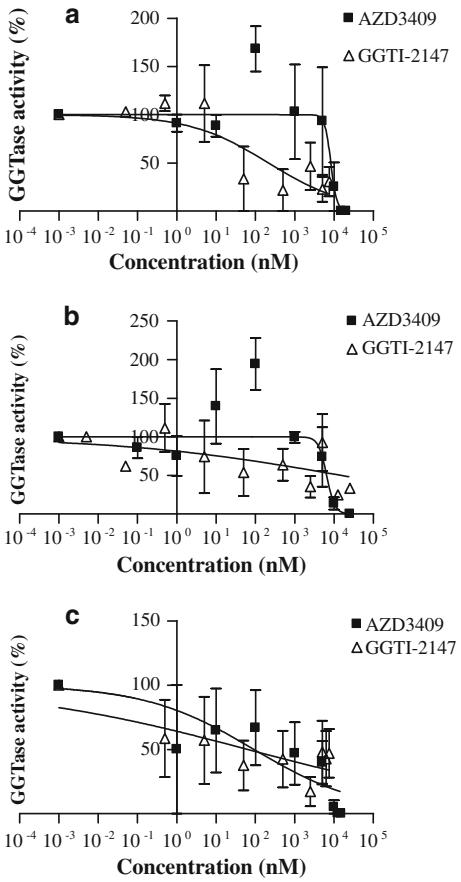

Fig. 5 GGTase activity in cells after treatment with either AZD3409 or GGTI-2147 at increasing concentrations (mean ± SEM). Data were fitted to a sigmoidal dose–response curve with a variable slope. The maximal effect (E_{max}) was set at 100%, the minimal effect was set at 0% (E_{min}). The following equation was used to describe the data: $y = (E_{min} + (E_{max} - E_{min}))/(1 + 10\exp(\log IC_{50} - x)\gamma)$. y = FTase activity relative to the basal level in untreated cells; x = logarithm of the drug concentration (nM). **a** MEF cells; AZD3409: $IC_{50} = 8{,}200$, $\gamma = -5.72$; GGTI-2147: $IC_{50} = 212$, $\gamma = -0.43$. **b** A549 cells; AZD3409: $IC_{50} = 6433$, $\gamma = -4.17$; GGTI-2147: $IC_{50} = 15517$, $\gamma = -0.15$. **c** MCF7 cells; AZD3409: $IC_{50} = 91.9$, $\gamma = -0.31$ GGTI-2147: $IC_{50} = 63.2$, $\gamma = -0.14$

and could also be important to obtain cytotoxicity in cancer cells. For future experiments, assessment of the effects of AZD3409 on signalling pathways downstream of Ras, e.g. MAPK and Akt, would be warranted.

Farnesylation is an important pharmacodynamic endpoint of FTIs. The mean IC_{50} for FTase activity of AZD3409 in cell lysates determined with a filter-binding assay was approximately 10 times higher than the IC_{50} determined in a functional assay. AZD3409 is a double prodrug and has to be converted to the active drug (AZD3409-acid) by esterases present in serum and in the cytoplasm. The rate and efficiencies of these metabolic reactions as well as the uptake efficiency of AZD3409-ester in cells might have a negative effect on the intracellular concentration of the AZD3409-acid. Consequently, the IC_{50} of AZD3409 in cells will be higher than the IC_{50} in a functional isolated enzyme inhibition assay. Measurement of the intracellular concentrations of AZD3490-ester and AZD3409-acid might enable the quantitative characterization of the metabolic processes. However, methods that are sensitive enough to detect intracellular concentrations of AZD3409-ester and AZD3409-acid are not yet available.

Besides the effect of AZD3409 treatment on FTase activity, we also determined the effect on the farnesylation at the total protein level and specifically at the HDJ-2 level. Both assays demonstrated inhibition of farnesylation in the three cell lines after drug administration. The FmC LC-MS/MS method is the first assay that has been described, which is able to quantify the absolute amount of farnesylated proteins. After lysis of cells, FmC is formed through cleavage by an endogenous protease. It is assumed that the amount of FmC that is formed after lysis correlates with the amount of cytosolic proteins that is farnesylated, since proteins are only farnesylated at the C-terminal cysteine. The FmC assay correlated well with the cellular FTase assay and the HDJ-2 western blot; a better inhibition of lonafarnib was observed in MCF-7 cells, while the difference in the inhibition profiles of both drugs in MEF and A549 cells was not significant. Previously, we concluded that HDJ-2 is a good biomarker for FTase activity [24]. Thus, these results suggest that also the FmC concentration in cell lysates can be used as a surrogate endpoint for FTase activity. Tandem mass spectrometry provides comparable

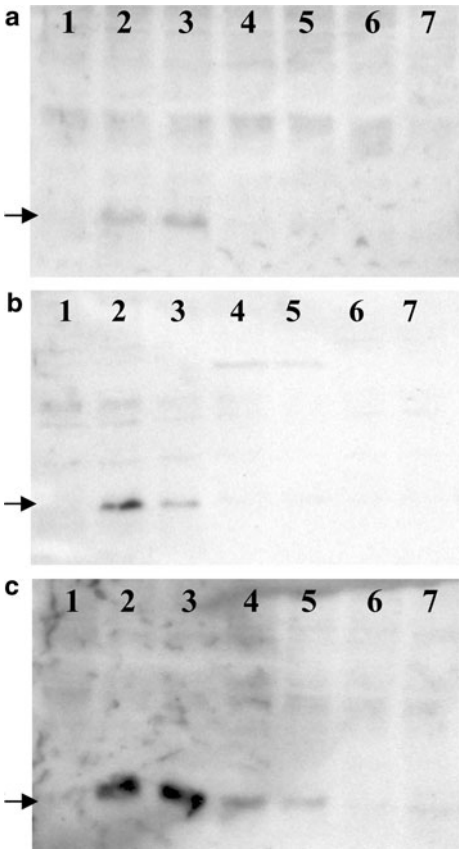

Fig. 6 Representative western blots of Rap1a. Appearance of a band at 22 kDa indicates inhibition of geranylgeranylation of Rap1a. **a** MEF cells. *1* blank; *2* 2.5 μM GGTI-2147; *3* 5 μM GGTI-2147; *4* 15 μM AZD3409; *5* 20 μM AZD3409; *6* 15 μM lonafarnib; *7* 20 μM lonafarnib. **b** A549 cells. *1* blank; *2* 2.5 μM GGTI-2147; *3* 5 μM GGTI-2147; *4* 5 μM AZD3409; *5* 10 μM AZD3409; *6* 25 μM lonafarnib; *7* 50 μM lonafarnib. **c** MCF-7 cells. *1* blank; *2* 5 μM GGTI-2147; *3* 7.5 μM GGTI-2147; *4* 12.5 μM AZD3409; *5* 15 μM AZD3409; *6* 12.5 μM lonafarnib; *7* 15 μM lonafarnib. All cells were incubated with the respective drug for 24 h

sensitivity and selectivity to the HDJ-2 western blot assay, but it is preferred because it makes absolute protein quantification possible, while western blot assays are only semi-quantitative. Despite the comparable results in all three biomarker assays, a clear correlation between the cytotoxicity and the inhibition of farnesylation was not found. To obtain in vitro proof of concept, longer continuous exposure studies might be required, since IC_{50} values in shorter assays may reflect an overlay of mechanisms of toxicity that are not due to the inhibition of farnesyl transferase.

Geranylgeranylation inhibition of AZD3409 was investigated using an GGTase-1 activity assay. Moreover, prenylation of Rap1a was determined as a surrogate endpoint.

Geranylgeranyl transferase-1 activity inhibition was achieved at much higher concentrations of AZD3409 than FTase inhibition in all cells. This is in accordance with the difference in IC_{50} values in a functional assay, in which AZD3409 is a potent inhibitor of FTase and a moderate inhibitor of GGTase-1. The lowest IC_{50} values were obtained in MCF-7 cells for both AZD3409 and GGTI-2147. Rap1a prenylation was not inhibited by AZD3409 in the MEF and A549 cell lines, while moderate inhibition was observed in the MCF-7 cells. As in both assays, the MCF-7 cell line was most sensitive to AZD3409, a correlation between inhibition of GGTase-1 and Rap1a prenylation has been shown. However, it might be necessary to improve the sensitivity of the assay as no unprenylated Rap1a could be detected in the MEF and A549 cells. Possibly, longer incubation times might reveal unprenylated Rap1a bands, although cytotoxicity could impose a problem. We conclude that Rap1a prenylation could serve as a biomarker for inhibition of GGTase activity by AZD3409. More insight into the GGTase-1 activity of AZD3409 in cells might be obtained with a quantitative LC-MS/MS method that determines the amount of geranylgeranylmethylcysteine (GGmC) in cell lysates. The high specificity of the mass spectrometer will allow a good discrimination between FmC and GGmC.

In summary, we have demonstrated that AZD3409 is a potent inhibitor of tumour cell growth and in vitro farnesylation and a moderate inhibitor of in vitro geranylgeranylation. Although AZD3409 did inhibit GGTase-1 in all cell lines, we could only demonstrate inhibition of Rap1a geranylgeranylation in the MCF-7 cell line.

Recommendation would include further experiments, preferably using quantitative assays, that provide more insight into the GGTase-1 activity of AZD3409 in cells. If significant GGTase-1 activity can be demonstrated in vitro and in vivo, the drug could be beneficial to patients with N- or K-Ras-driven tumours, which probably require inhibition of both farnesylation and geranylgeranylation to attain cell growth arrest. Phase I studies that include well-defined biomarker assays are required to establish clinical proof of concept.

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